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Two novel calcium-binding proteins from cytoplasmic granules of the protozoan parasite *Entamoeba histolytica*

Rosa Nickel[a], Thomas Jacobs[a], Britta Urban[a,1], Henning Scholze[b], Heike Bruhn[a], Matthias Leippe[a,*]

[a] *Department of Molecular Biology, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany*
[b] *Department of Biology/Chemistry, University of Osnabrück, Osnabrück, Germany*

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Abstract We report on the molecular characterisation of two novel granule proteins of the protozoon and human pathogen *Entamoeba histolytica*. The proteins, which were named grainin 1 and 2, show a considerable structural similarity to calcium-binding proteins, particularly within EF-hand motifs. Each grainin possesses three of these putative calcium-binding sites. Based on careful inspection of known structures of protein families containing EF-hands, a domain of grainin 1 covering two EF-hand motifs was modeled by homology. Calcium-binding activity of grainins was demonstrated by two independent methods. These granule proteins may be implicated in functions vital for the primitive phagocyte and destructive parasite such as control of endocytotic pathways and granule discharge.

Key words: Calcium-binding protein; EF-hand; Cytoplasmic granule; *Entamoeba histolytica*

1. Introduction

The protozoon *Entamoeba histolytica* inhabits the lower intestine of man and uses bacteria as a major nutrient source. For unknown reasons, amoebae may become invasive, penetrate the mucosa, destroy host tissues and infiltrate extraintestinal organs leading to severe abscesses, mainly of the liver. As the etiologic agent of human amoebiasis, *E. histolytica* causes morbidity and mortality worldwide. Due to the fact that invasion into host tissues is not an obligatory part of the life cycle of the amoeba [1], it has been postulated repeatedly that the effective armament which kill phagocytozed bacteria and destroy host cells and tissues may overlap and is maintained by coincidental selection [2–4].

Morphologically, *Entamoeba* is a eukaryotic cell which lack many characteristic organelles such as mitochondria, golgi apparatus and rough endoplasmatic reticulum, centrioles and microtubules but is equipped with an enormous amount of vacuoles and cytoplasmic granules [5]. It has been calculated that vesicular organelles occupy about 40% of the total cell volume [6]. Amoebae kill a variety of host cells in a rapid and contact-dependent cytolytic reaction in which they are considered to transfer cytolytic molecules to the target cell membrane upon granule exocytosis. Therefore, the granular vesicles have been viewed as functional equivalents of both the lysosomes and the cytotoxic vesicles of higher eukaryotic cells [6]. Inside amoebic granules potent factors such as lysozymes [7], collagenase [8], phospholipases [9], cysteine proteinases [10] and the pore-forming amoebapores [11] have been found. This prompted us to further analyse the molecular repertoire of these lysosome-like organelles.

Here, we describe the purification of two granule proteins, the molecular cloning of which revealed that they both are putative calcium-binding proteins as they each contain three of the EF-hand motifs typical for this kind of proteins. The ability of the proteins to bind calcium was proven by biochemical assays. Ionic calcium play a crucial role in numerous cellular functions such as aggregation, adhesion, protein secretion and membrane dynamics in endocytosis. In amoebae, it is thought to be involved in the induction of granule exocytosis and the subsequent release of hydrolytic enzymes and cytolytic proteins thereby triggering cytopathic effects and cytolysis of host cells [12]. Therefore, the two novel granular calcium-binding proteins may play a crucial role in these processes.

2. Materials and methods

2.1. Cultivation and harvesting of E. histolytica

Trophozoites of the pathogenic *E. histolytica* isolate HM-1:IMSS were cultured axenically in medium TYI-S-33 in plastic tissue culture flasks [13]. Trophozoites from cultures in late-logarithmic phase were harvested after being chilled on ice for 10 min, sedimented at $430\times g$ for 3 min at 4°C and washed three times in ice-cold phosphate-buffered saline.

2.2. Purification of the calcium-binding protein

Freshly harvested and washed amoebae (5×10^8) were lysed by nitrogen cavitation and the cytoplasmic granules were enriched by differential centrifugation [11]. The granules were extracted in 10 ml of 50 mM sodium acetate, pH 4.5, containing 150 mM NaCl by three cycles of sonification (70 W for 30 s) and centrifuged at $150\,000\times g$ for 1 h at 4°C. The resulting supernatant was passed over a Superdex G 75 prep grade 26/60 column (Pharmacia) using the extraction buffer as eluent. Each fraction was tested for inhibition of bacterial growth and lysozyme activity [7], cysteine proteinase activity [14], pore formation [15] and phospholipase A2 activity [16]. The material representing the maximum of phospholipase activity was loaded on a Mono S HR 10/10 cation-exchange column (Pharmacia LKB) after dilution with three volumes of starting buffer (50 mM sodium acetate, pH 4.5). Adsorbed protein was eluted by washing with the same

*Corresponding author: Fax: (49)-40-42818 512.
E-mail: leippe@bni.uni-hamburg.de

[1] Present adress: Institute for Molecular Medicine, John Radcliffe Hospital, Oxford, UK.

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buffer (40 ml) and by use of an NaCl gradient from 0 to 500 mM (150 ml) and a final wash of 1 M NaCl (40 ml). Active fractions were pooled, diluted with two volumes of the aforementioned starting buffer and applied to a Mono S HR 5/5 column (Pharmacia LKB) equilibrated with starting buffer. The column was washed with the same buffer (5 ml) and developed with a 20 ml gradient of 150–450 mM NaCl. Two prominent proteins were finally purified by reversed-phase high performance liquid chromatography (RP-HPLC) performed on an Aquapore Butyl 300 column (2.1×30 mm; Brownlee Labs) connected with a 130 A separation system (Applied Biosystems). Elution was done with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid at 50°C over 45 min. A flow rate of 0.2 ml min^{-1} was applied, the effluent was monitored by absorbance at 214 nm and 0.2 ml fractions were collected.

2.3. Protein analysis

Tricine–SDS–PAGE was performed according to Schägger and von Jagow [17]. The protein concentration of samples was estimated by measuring absorbance at 280 nm using bovine serum albumin as standard. For N-terminal sequencing, peak fractions from analytical RP-HPLC were collected manually and analysed using a gas-phase protein sequencer (model 437 A; Applied Biosystems).

2.4. Molecular cloning

Specific cDNA fragments for each protein were amplified from a λZap cDNA library of the *E. histolytica* isolate HM-1:IMSS using degenerated sense oligonucleotides (5′-CAAGC$^{T}/_{A}$GATCCACTT-ATTCAAAG for grainin 1; 5′-GATCCAAAT$^{C}/_{T}$T$^{T}/_{A}$AAATT$^{T}/_{C}$C-AATGG for grainin 2) deduced from the respective N-terminal amino acid sequences together with a poly-dT-oligonucleotide. Polymerase chain reaction (PCR) fragments were sequenced on both strands. Primer extension experiments for grainin 1 using an antisense oligonucleotide (5′-GTTCTTTAAATTCAGTTCCTG) were performed and the extension product was determined according to the anchored PCR technique [18]. The 5′-end of the cDNA coding for grainin 2 was amplified from the cDNA library of *E. histolytica* using an antisense oligonucleotide (5′-CAACTAATGGGAACCACC) together with M13 reverse primer.

2.5. Calcium-binding assay

Proteins were electroblotted on a PVDF membrane by the semidry blotting technique with 10 mM CAPS, pH 11.0/10% methanol under non-reducing conditions. Subsequent incubation with the fluorescent probe Quin 2 and washing procedures were carried out as described [19].

2.6. Fast purification protocol

Grainins were enriched according to the method of Gerke et al. [20]. Amoebae were lysed in buffer A (150 mM NaCl, 10 μM E-64, 10 mM $CaCl_2$, 10 mM imidazole, pH 7.4) at 4°C for 20 min and centrifuged at 16 000×*g* at 4°C for 15 min. Precipitated material was resuspended in buffer B (100 mM KCl, 10 μM E-64, 1 mM $CaCl_2$, 1% Triton X-100, 0.5 mM dithiothreitol (DTT), 10 mM imidazole, pH 7.4), stirred at 4°C for 30 min and centrifuged as described before. This latter step was repeated three times. Finally, the pellet was solubilised in buffer C (100 mM KCl, 5 mM EGTA, 0.5 mM DTT, 10 mM imidazole, pH 7.4) followed by centrifugation. The supernatant contains predominantly the grainins. The proteins remained soluble during dialysis against 10 mM Tris, pH 7.8, whereas they can easily be precipitated in the presence of 1 mM $CaCl_2$. The dialysed proteins were purified further by anion-exchange chromatography on Mono Q (Pharmacia) and subjected to bromcyan cleavage followed by protein sequencing of separated fragments [21].

2.7. Homology modeling

Three different template structures were combined to model by homology the first domain of grainin 1 comprising its first two EF-hands. Regarding the calcium-coordinating loop, the most identical structures were identified by sequence analysis. A textword search of the PDB entries (http://www.rcsb.org/pdb/) revealed all known structures containing EF-hand motifs. Using the program FINDPATTERN (GCG-package, [22] the corresponding sequences were searched for the motif DXDXSX{6,6}E specifying all the calcium-coordinating amino acid residues of the EF-hand motifs of grainin 1 except the amino acid in position 7 which contributes with its main chain carbonyl oxygen to the ion coordination. Analyzing the amino acid identity in the matching sequences led to the choice of the first motif of whiting parvalbumin (1a75.pdb) and the third motif of the calcium-binding domain of porcine calpain (1alv.pdb) as template structures of the first and second EF-hand motif of grainin 1, respectively. The structures of representatives of all known protein families containing EF-hand motifs found in the PDBsum database (http://www.biochem.ucl.ac.uk/bsm/pdbsum/) were carefully investigated regarding the exact length of secondary structure elements in the EF-hand domains in comparison to the predicted one of grainin 1 (PHD program, EMBL, Heidelberg, Germany). This analysis suggested the most similar second domain of troponin C (1aj4.pdb) as suitable template for the modeling of the remaining parts of the grainin 1 domain. The modeling was performed using the program WHATIF [23].

3. Results and discussion

3.1. Purification of two novel granular proteins

Amoebic cytoplasmic granules were enriched by differential centrifugation after subjecting the amoebae to nitrogen cavitation. The extract of the granules containing 120 mg protein was fractionated by molecular sieve chromatography. At a position corresponding to approximately 20 to 40 kDa, material was eluted which exerted lysozyme, cysteine proteinase, and phospholipase A2 activity (not shown). Further purification by Mono S-cation exchange chromatography yielded a fraction that eluted at 300 mM NaCl and exerted high phospholipase A2 activity. Upon SDS–PAGE two prominent, closely spaced protein bands appeared at positions of approximately 22 and 23 kDa. Rechromatography on Mono S and analysis of single fractions revealed that the 22/23 kDa proteins nearly coeluted with phospholipase activity but did not display such activity by itself. These two granule proteins which appear to have several physical properties in common were separated in a final step by analytical RP-HPLC and yielded apparently homogeneous material as judged by SDS–PAGE (Fig. 1). Under reducing conditions both proteins migrated as having a molecular mass of 23 kDa indicating the presence of intramolecular disulphide bonds of the smaller entity (not shown).

3.2. Protein sequencing and molecular cloning

Automated Edman degradation of the first peak representing the 23 kDa protein of the column eluate yielded sequence information of 29 residues (see Fig. 1) of which the first 19 residues were confirmed by a second trial. Sequence analysis of the second maximum confirmed the purity of the 22 kDa protein also and indicated that it is clearly distinct from the 23 kDa protein; it allowed identification of the N-terminus up to residue 16 (Fig. 1), of which in particular the first nine had an unambiguous attribution. In the following the 22 kDa and the 23 kDa protein from the granules were tentatively named grainin 1 and grainin 2, respectively.

cDNA cloning and the deduced amino acid sequence revealed the complete primary structure of both proteins. The N-terminal amino acid residues determined are each preceeded by a stretch of hydrophobic amino acid residues which may serve as a signal peptide necessary for the transport of grainins into vesicles. The amino acid sequence identity between the two isoforms is 49%, and with respect to amino acid residues with similar properties the similarity is approximately 64% (Fig. 2). Grainin 1 and 2 each contain in their primary structure three consensus metal-ligating loop sequences found within a helix–loop–helix structure termed EF-hand. The EF-

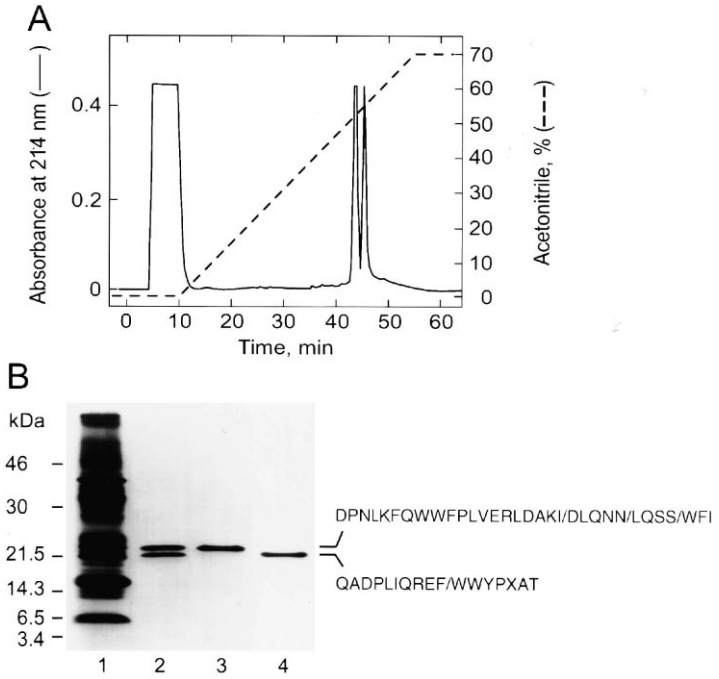

Fig. 1. Initial purification of grainins. Final purification of grainins was achieved by RP-HPLC (A). The silver-stained SDS-gel (B) represents the different stages of purification: Lane 1, acid extract of amoebic granules; lane 2, fraction obtained after two consecutive cation-exchange chromatographies; lane 3 and 4, the first and second peak of the RP-HPLC, respectively. The N-terminal amino acid sequences obtained by Edman degradation of the purified proteins are shown at the right.

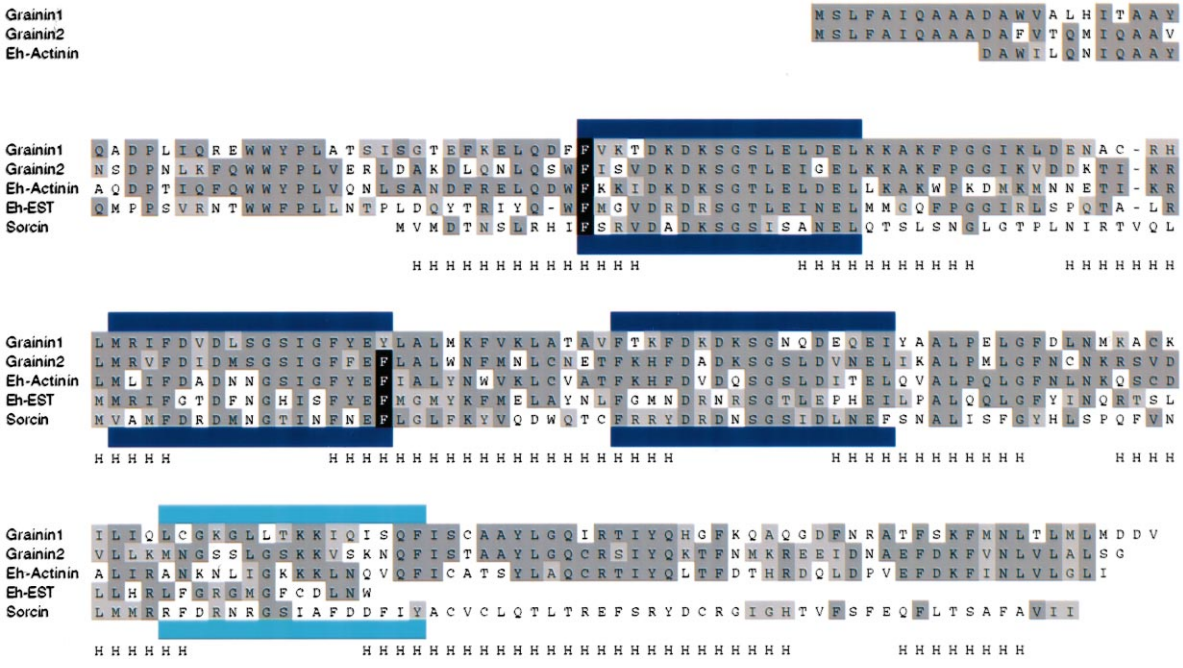

Fig. 2. Structural similarity of grainins to other proteins. The amino acid sequences of grainin 1 and 2 were aligned to an actinin-like protein (Eh-actinin) and an EST of *E. histolytica* (Eh-EST), and sorcin of *S. japonicum* (sorcin). Amino acid residues identical to one of the grainins are shaded dark grey and amino acid residues with similar properties are shaded light grey. Gaps were introduced to maximise alignment. The EF-hand motifs are marked in blue, while the supplemental ancestral motif which lost the capability of calcium-binding is marked in light blue. Phenylalanine residues conserved in functional EF-hand motifs [28] are shown in white letters on a black background. α-Helical structures within the grainins were predicted using the PHD secondary structure prediction program (EMBL, Heidelberg, Germany) and are indicated by H below the sequences. The nucleotide and amino acid sequences are available from the EMBL/GenBank/DDBJ databases under the accession numbers AF085196 (grainin 1), AF082530 (grainin 2), AF208390 (Eh-actinin), AB002727 (Eh-EST) and U39069 (sorcin).

hand is a recurring motif in calcium-binding proteins, of which calmodulin with four EF-hands is a well-known example by serving as a calcium sensing protein in nearly all eukaryotic cells [24]. In the grainins, a supplemental ancestral EF-hand motif which lost the capability of calcium-binding can be identified. Such a motif is present in most of the other proteins containing an odd number of EF-hand motifs.

An overall amino acid sequence identity between 35 and 50% for grainins was found to the putatively calcium-binding region of an actinin-like protein and an amino acid sequence deduced from an EST clone (Fig. 2), both representing until now uncharacterised gene products of *E. histolytica*. With regard to all known natural calcium-binding proteins, only a weak sequence identity of 20 and 23% for grainin 1 and 2, respectively, to sorcin of *Schistosoma japonicum* was found, which is mainly due to homology in the three EF-hand motif regions of the proteins.

Both grainins exhibit only moderate sequence identity to the *Entamoeba* calcium-binding protein EhCaBP described and characterised in detail for this organism previously [25,26]. Whereas the newly recognised proteins described here appear to be localised within the amoebic granules, EhCaBP is a cytosolic protein.

3.3. Calcium-binding properties

The calcium-binding activity of the grainins were confirmed by two independent methods. The detection of calcium ions bound by the electroblotted proteins using the fluorescent calcium-chelating agent Quin 2 [19] clearly demonstrates their calcium-binding ability (Fig. 3).

Furthermore, the observation that highly enriched grainins are precipitated by calcium-containing buffers and are easily resolubilised in the presence of EGTA implicates a calcium-binding capability. This procedure is also a suitable and fast method for purification of the proteins from crude amoebic extracts by simply lysing the trophozoites in a calcium-containing buffer. The predominant proteins in the resulting precipitate are grainin 1 and 2 as evidenced by migration upon SDS–PAGE and by sequencing of fragments obtained by bromcyan cleavage (Fig. 3). The failure of determining the N-termini of the precipitated proteins directly and also the fact that the sequence of a fragment of grainin 2 (IQAAVNTDPN)) reaches beyond the N-terminus determined originally suggest that a peptidase has acted on the proteins during the initial multi-step purification procedure.

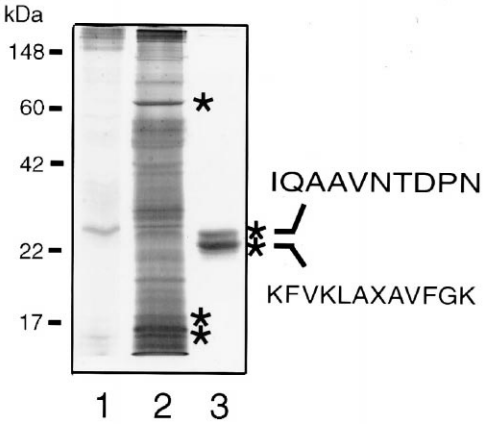

Fig. 3. Fast purification of grainins by calcium precipitation and detection of protein-bound calcium. The silver-stained SDS-gel shows the different fractions obtained by the fast purification protocol: lane 1, proteins precipitated in the presence of calcium; lane 2, supernatant containing soluble proteins; lane 3, proteins shown in lane 1 resolubilised in a buffer containing EGTA. The asterisks indicate calcium-binding proteins detected by the fluorescent quinolin calcium-indicator Quin 2. The N-termini shown at the right are from fragments generated by bromcyan cleavage of the proteins shown in lane 3 identifying them as grainin 1 and 2.

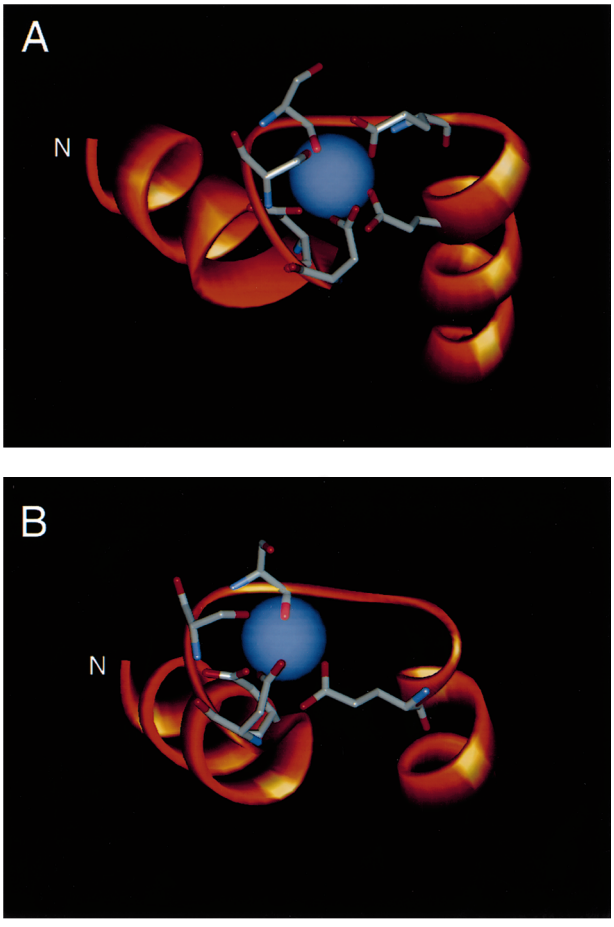

Fig. 4. Calcium-coordinating amino acid residues of the first two EF-hand motifs of grainin 1. The calcium-binding loop of EF-hand motif 1 (A) and motif 2 (B) is shown in yellow with bound calcium ions shown in transparent grey. The calcium-coordinating amino acid residues are exposed.

3.4. Comparative modeling

The first domain of grainin 1 consisting of two EF-hand motifs was modeled by homology. The structural templates were chosen by careful investigation of the known structures of protein families containing EF-hand motifs. In a typical EF-hand motif, the calcium ion is coordinated by an oxygen of the five amino acid residues marked in bold (PROSITE pattern PS00018, [27]): **D**-X-**(DNS)**-(LYFVW)-**(DENSTG)**-(DNQGHRK)-**{GP}**-(LIYMC)-(DENQSTAGC)-X-X**(ED)**-(LIYMFVW).

The calcium-binding loops were modeled on templates most identical regarding the calcium-coordinating amino acid residues: whiting parvalbumin and porcine calpain for motif 1 and motif 2, respectively. In the first motif of whiting parvalbumin as well as in motif 1 of grainin, a glutamate residue in

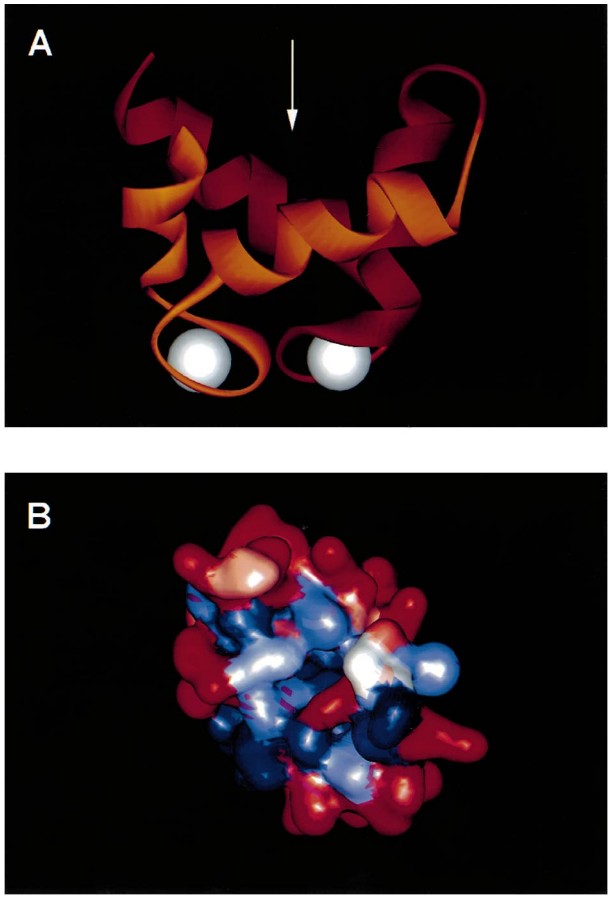

Fig. 5. The modeled structure and protein surface of the first calcium-binding domain of grainin 1. The first EF-hand motif is shown in red (A), the second one in yellow and bound calcium ions are shown in white. The protein surface of the side opposite to the calcium ions coloured according to hydrophobicity is presented in (B). The direction of the view is indicated by the white arrow in (A). Blue means hydrophobic amino acid residues, red means hydrophilic ones.

position 9 of the motif occupies the sixth coordinating site (Fig. 4) whereas in most of the other known structures this function is fulfilled by a water molecule. The motif 2 of grainin is nearly identical to the third calcium-binding site of the calcium-binding domain of porcine calpain, except that the serine in the motif position 7 is changed to threonine in grainin 1, but this residue binds the calcium ion only with the main chain carbonyl oxygen (Fig. 4). The secondary structure prediction of the grainin 1 domain resembles the known secondary structure of the second domain of troponin C. Therefore, we combined the modeled calcium-binding loops with the model of the remaining parts of the domain on the template of chicken cardiac troponin C (Fig. 5). The EF-hands are presented in an open conformation, thereby exposing the hydrophobic residues on the side opposite the calcium-binding loops (Fig. 5) which may suggest a protein-binding capability. Many regulatory calcium-binding proteins obtain the open conformation, which enables protein–protein interactions in response to calcium-binding. In the known structures, ancestral EF-hand motifs retain the helix–loop–helix structure. Thus, the second domain of grainin 1 covering the third and the non-binding ancestral motif is predicted to adopt the typical structure of a domain of two EF-hands. Due to the relatively short helix of 13 amino acid residues in between the two domains, these are not structurally independent like the domains in the dumbbell-shaped calmodulin or troponin C. The overall structure of the whole grainin 1 molecule appears to be compact and globular. Although numerous functions may be hypothesised for the grainins, a most plausible function would be the control of endocytotic pathways and granular discharge in dependence upon calcium concentration. Particularly the latter cellular mechanism is a prerequisite for the secretion of tissue-destructive molecules and as such determines pathogenic behaviour of the parasite.

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References

[1] Clark, C.G. (1995) Trop. Dis. Bull. 92, R147–R155.
[2] Horstmann, R.D., Leippe, M. and Tannich, E. (1992) Trop. Med. Parasitol. 43, 213–218.
[3] Leippe, M. (1997) Parasitol. Today 13, 178–183.
[4] Gosh, S.K. and Samuelson, J. (1997) Infect. Immun. 65, 4243–4249.
[5] Espinosa-Cantellano, M., Gonzales-Robles, A., Chavez, B., Castan on, G., Arguello, C., Lazaro-Haller, A. and Martinez-Palomo, A. (1998) J. Eukaryot. Microbiol. 45, 265–272.
[6] Bakker-Grunwald, T. (1991) in: Biochemical Protozoology (Coombs, G. and North, M., Eds.), pp. 367–376, Taylor and Francis, London.
[7] Jacobs, T. and Leippe, M. (1995) Eur. J. Biochem. 231, 831–838.
[8] Serrano, J.J., de la Garza, M., Moreno, M.A., Tovar, R., Leon, G., Tsutsumi, V. and Munoz, M.L. (1994) Mol. Microbiol. 11, 787–792.
[9] Vargas-Villarreal, J., Olvera-Rodriguez, A., Mata-Cardenas, B.D., Martinez-Rogriguez, H.G., Said-Fernandez, S. and Alagon-Cano, A. (1998) Parasitol. Res. 84, 310–314.
[10] Jacobs, T., Bruchhaus, I., Dandekar, T., Tannich, E. and Leippe, M. (1998) Mol. Microbiol. 27, 269–276.
[11] Leippe, M., Andrä, J., Nickel, R., Tannich, E. and Müller-Eberhard, H.J. (1994) Mol. Microbiol. 14, 895–904.
[12] Long-Krug, S.A. and Ravdin, J.I. (1988) in: (Ravdin, J.I., Ed.), pp. 251–262, Wiley, New York.
[13] Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) Trans. R. Soc. Med. Hyp. 72, 431–432.
[14] Leippe, M., Sievertsen, H.J., Tannich, E. and Horstmann, R.D. (1995) Parasitology 111, 569–574.
[15] Leippe, M., Ebel, S., Schoenberger, O.L., Horstmann, R.D. and Müller-Eberhard, H.J. (1991) Proc. Natl. Acad. Sci. USA 88, 7659–7663.
[16] Ghomashchi, F., O'Hare, T., Clary, D. and Gelb, M.H. (1991) Biochemistry 30, 7298–7305.
[17] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[18] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[19] Tatsumi, R., Shimada, K. and Hattori, A. (1997) Anal. Biochem. 254, 126–131.
[20] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[21] Matsudeira, P. (1990) Methods Enzymol. 182, 602–613.
[22] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[23] Vriend, G. (1990) J. Mol. Graph. 8, 52–56.
[24] Chin, D. and Means, A.R. (2000) Trends Cell Biol. 10, 322–328.
[25] Prasad, J., Bhattaharya, S. and Bhattacharya, A. (1992) Mol. Biochem. Parasitol. 52, 137–140.
[26] Yadava, N., Chandok, M.R., Prasad, J., Bhattacharya, S., Sopory, S.K. and Bhattacharya, A. (1997) Mol. Biochem. Parasitol. 84, 69–82.
[27] Bairoch, A., Bucher, P. and Hofmann, K. (1997) Nucleic Acids Res. 25, 217–221.
[28] Hooman, H.R., Bauer, M., Patterson, J. and Smith, D.W. (1999) J. Mol. Microbiol. Biotechnol. 1, 175–182.